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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*Axordia Limited
Firth Court
SHEFFIELD
S10 2TN
GB

08259160001

Patents ADP number *(if you know it)*

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4. Title of the invention

Hyperproliferation

5. Name of your agent *(if you have one)*

Harrison Goddard Foote

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*31 St Saviourgate
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HYPERPROLIFERATION

The invention relates to a method to inhibit cell proliferation in diseases characterised by hyperproliferation, typically but not exclusively, psoriasis, comprising introducing inhibitory RNA (RNAi) into a cell to ablate mRNA's which
5 encode polypeptides involved in essential cell processes; vectors encoding said RNAi; and compositions comprising said vectors or RNAi.

A number of techniques have been developed in recent years which purport to
10 specifically ablate genes and/or gene products. For example, the use of antisense nucleic acid molecules to bind to and thereby block or inactivate target mRNA molecules is an effective means to inhibit the production of gene products. This is typically very effective in plants where anti-sense technology produces a number of striking phenotypic characteristics. However, recombinant antisense technology is
15 variable leading to the need to screen many, sometimes hundreds of, transgenic organisms carrying one or more copies of an antisense transgene to ensure that the phenotype is indeed truly linked to the antisense transgene expression. Antisense techniques, not necessarily involving the production of stable transfectants, have been applied to cells in culture, with variable results.

20

In addition, the ability to be able to disrupt genes via homologous recombination has provided biologists with a crucial tool in defining developmental pathways in higher organisms. The use of mouse gene "knock out" strains has allowed the dissection of gene function and the probable function of human homologues to the deleted mouse
25 genes, (Jordan and Zant, 1998).

A much more recent technique to specifically ablate gene function is through the introduction of double stranded RNA, also referred to as inhibitory RNA (RNAi), into a cell which results in the destruction of mRNA complementary to a sequence
30 included in the RNAi molecule. The RNAi molecule comprises two complementary strands of RNA (a sense strand and an antisense strand) annealed to each other to

form a double stranded RNA molecule. The RNAi molecule is typically derived from an exonic sequence of the gene which is to be ablated.

5 Recent studies suggest that RNAi molecules ranging from 19-1000bp derived from coding sequence are effective inhibitors of gene expression. Surprisingly, only a few molecules of RNAi are required to block gene expression which implies the mechanism is catalytic.

10 The exact mechanism of RNAi action is unknown although there are theories to explain this phenomenon. For example, all organisms have evolved protective mechanisms to limit the effects of exogenous gene expression. For example, a virus often causes deleterious effects on the organism it infects. Viral gene expression and/or replication therefore needs to be repressed. In addition, the rapid development of genetic transformation and the provision of transgenic plants and animals has led
15 to the realisation that transgenes are also recognised as foreign nucleic acid and subjected to phenomena variously called quelling (Singer and Selker, 1995), gene silencing (Matzke and Matzke, 1998), and co-suppression (Stam et. al., 2000).

Initial studies using RNAi used the nematode *Caenorhabditis elegans*. RNAi
20 injected into the worm resulted in the disappearance of polypeptides corresponding to the gene sequences comprising the RNAi molecule (Montgomery et. al., 1998; Fire et. al., 1998). More recently the phenomenon of RNAi inhibition has been shown in a number of eukaryotes including, by example and not by way of limitation, plants, trypanosomes (Shi et. al., 2000) *Drosophila spp.* (Kennerdell and Carthew, 2000).
25 Recent experiments have shown that RNAi may also function in higher eukaryotes. For example, it has been shown that RNAi can ablate *c-mos* in a mouse oocyte and also E-cadherin in a mouse preimplantation embryo (Wianny and Zernicka-Goetz, 2000).

A number of diseases are characterised by uncontrolled cell division, or hyperproliferation, which results in disease. Examples include, psoriasis, cancer and viral diseases which result in cell transformation.

- 5 Psoriasis is a generic term to cover a range of diseases characterised by abnormal proliferation of skin cells. The disease covers the following list which is not exhaustive but merely illustrative: nail psoriasis; scalp psoriasis; plaque psoriasis; pustular psoriasis; guttate psoriasis; inverse psoriasis; erythrodermic psoriasis; psoriatic arthritis.

10

- Psoriasis is one of the most frequent skin diseases, affecting 1-3% of the Caucasian population world wide. The disease is characterised by alterations in a variety of different cell types. These include epidermal keratinocytes which are characterised by hyperproliferation and an altered differentiation which is indicated by focal
15 parakeratosis and aberrant expression of keratinocyte genes encoding hyperproliferation-associated keratin pair 6/16, involucrin, filaggrin, and integrin adhesion molecules (e.g. VLA-3, 5, 6).

- Current methods to control psoriatic conditions include the use of topical
20 applications of coal tar which reduce itching and scaling of skin. However, coal tar sensitises skin to ultraviolet thereby rendering individuals susceptible to sunburn. Treatment with coal tar can also result in photosensitivity. An alternative to the use of coal tar is topical steroids. Although effective, steroid treatment can result in thinning of skin. Also if steroids are used long term the body can become resistant
25 thereby rendering the treatment ineffective. Other pharmaceutical treatments include the topical application of anthralin, vitamin D3 and retinoid treatment. Oral medications are also available to those with severe forms of the disease which do not respond well to topical treatments. These include methotrexate, cyclosporins, Tegison. Each of these medications have serious side effects which include liver and
30 lung damage (methotrexate), immunosuppression (cyclosporins) and rashes, hair loss and hepatitis (Tegison). Also many of these drugs are incompatible with pregnancy

and therefore should be avoided by women of childbearing age. Clearly there is a need for alternative treatments which do not have the above identified disadvantages.

When normal keratinocytes are cultured, they assume a hyperproliferative state that is similar to psoriasis *in vivo* and has been labelled the pseudo-psoriatic phenotype. This provides an excellent model of testing various therapeutic treatments *in vitro* before animal model experiments are undertaken. Moreover, there exists an animal model for psoriasis which allows the testing of various therapies with respect to the treatment of this condition, see US 5, 945, 576 which is incorporated by reference.

The majority of cells (>95%) which comprise skin are keratinocytes at various stages of differentiation. Keratinocytes of the basal layer are constantly dividing and daughter cells subsequently move outwards, during which they undergo a period of differentiation and arrest cell division. It is the uncontrolled division of these keratinocytes which result in the formation of psoriatic plaques.

Teratomas are a form of germ cell tumour, containing teratocarcinoma cells (EC cells). These cells have many features in common with ES/EG cells. The most important of these features is the characteristic of pluripotentiality.

Teratomas contain a wide range of differentiated tissues, and have been known in humans for many hundreds of years. They typically occur as gonadal tumours of both men and women. The gonadal forms of these tumours are generally believed to originate from germ cells, and the extra gonadal forms, which typically have the same range of tissues, are thought to arise from germ cells that have migrated incorrectly during embryogenesis. Teratomas are therefore generally classed as germ cell tumours which encompasses a number of different types of cancer. These include seminoma, embryonal carcinoma, yolk sac carcinoma and choriocarcinoma. This in turn will reduce or eliminate the ability of these tumour to grow and hence metastasize.

A critical gene controlling pluripotency in Human Embryonal carcinoma and Embryonic Stem cells is Oct-4 (Nichols et. al., 1998). Changes in expression of Oct4 result in the differentiation of pluripotent cells (Niwa et. al., 2000)

5 The elimination of *Oct4* protein expression results in the reduction in growth and differentiation of nullipotent and pluripotent human embryonal carcinoma (EC) cells, the malignant counterparts of ES cells derived from testicular germ cell tumours, as well as human ES cells themselves. This should allow the malignant cells to be targeted, causing both growth reduction and the differentiation of the malignant cells that make up germ cell tumours.

10

The elimination of *Oct4* protein expression in germ cell tumours could be achieved by dsRNA against *Oct4*.

15 In differentiating cultures of embryonic stem cells there is the chance for ES cells to remain undifferentiated as contaminating cells. These cells may present problems at a later stage if the cells are to be used for transplantation, due to their pluripotency and increased growth rate relative to the differentiated derivatives which could result in teratoma formation in recipients. Targeting *Oct4* mRNA using RNAi in these cultures would result in the elimination of contaminating cells thus improving the
20 safety of ES cells as a medical treatment.

Furthermore the use of dsRNA against *Oct4* to render germ cells in animals incapable of producing gametes which will make an animal sterile.

25 The MHC class I molecules are produced from the HLA-A, -B, -C loci and interact with beta-2-microglobulin. Variants such as HLA-E are expressed on MHC class I expressing cells after interaction with the MHC class I proteins (Lee et. al., 1998). Cytotoxic T cells can recognise MHC-I presenting cells and induce cytotoxicity. It would be desirable to be able to modulate the immune response of stem cells,
30 particularly embryonic stem cells, so that they are tolerated by the host. This would involve delivering dsRNA *in vivo* to cells to protect against an immune response.

Furthermore, cells treated with dsRNA *in vitro* against beta-2- microglobulin or type one HLA complex genes would be less immunogenic this may enable immunotolerable cells to be generated prior to transplantation. Transient introduction of dsRNA to generate an RNAi effect may give the transplanted cells a selective advantage in the patient and could be used in combination of immunosuppression through drugs or the generation of a chimeric immune system. The use of recombinant DNA constructs that enable the manufacture of dsRNA (including hairpin RNA) would enable the production of permanently MHC-1 suppressed cells thus facilitating long term engraftment of cells and tissue. Specific lengths of dsRNA which may be mentioned, include, *inter alia*, 19, 20 and 21 bps.

According to a first aspect of the invention there is provided a method to inhibit cell division comprising introducing at least one inhibitory RNA molecule (RNAi) into a hyperproliferative cell wherein said RNAi molecule inhibits the expression of at least one gene which mediates at least one essential process in said cell.

In a preferred method of the invention said RNAi molecule inhibits at least one step in the cell-division-cycle of said cell.

The term "hyperproliferative" refers to a diseased cell which shows an uncontrolled cell-division-cycle resulting in a disease condition, for example psoriasis or cancer. It will be apparent to one skilled in the art that the introduction of RNAi molecules into a cell may be by addition of a RNAi molecule to a cell in a composition or by the provision of gene therapy vectors adapted to express both sense and antisense nucleic acids thereby producing a RNAi molecule *in situ*.

In a yet further preferred method of the invention said hyperproliferative cell is a keratinocyte, preferably a psoriatic keratinocyte.

In a yet further preferred method of the invention said hyperproliferative cell is an embryonal carcinoma cell (EC cell).

In a yet further preferred method of the invention said hyperproliferative cell is an embryonal stem cell (ES cell).

- 5 In a further preferred method of the invention of the invention said sequence comprises at least one of the sequences, or part thereof, selected from the group consisting of: hyperproliferation-associated keratin pair 6/16, involucrin, filagrin, integrin adhesion molecules (e.g. VLA-3, 5, 6), *Oct4* or variants thereof.
- 10 According to a further aspect of the invention there is provided a method to inhibit an immune response comprising introducing at least one inhibitory RNA molecule (RNAi) into a cells wherein said RNAi molecule inhibits the expression of at least one gene which mediates at least one immunogenic response to said cell.
- 15 In a yet further preferred method of the invention said cell is a stem cell, preferably said stem cell is embryonic.

In a more preferred embodiment said HLA gene is beta-2-microglobulin, a low molecular weight component of HLA antigens.

20

In a still further preferred method of the invention said gene is a type I HLA complex gene, selected from the group consisting of HLA-A, B, C, E, F, G.

- 25 According to a further aspect of the invention there is provided an RNAi molecule characterised in that it comprises the coding sequence of at least one gene which mediates at least one essential process in at least one hyperproliferative cell.

In a preferred embodiment of the invention the essential process is at least one step in the cell-division-cycle.

30

In a preferred embodiment said coding sequence is an exon.

Alternatively said RNAi molecule is derived from intronic sequences or the 5' and/or 3' non-coding sequences which flank coding/exon sequences of genes which mediate said essential process.

5

In a further preferred embodiment of the invention the length of the RNAi molecule is between 10bp-1000bp. More preferably still when the length of RNAi is in the range of 100-1000bp the length is selected from 100bp; 200bp; 300bp; 400bp; 500bp; 600bp; 700bp; 800bp; 900bp; or 1000bp. More preferably still said RNAi is at least 1000bp. More preferably still when the length of RNAi is in the range of 10-1000bp the length is preferably from 10-100bp and may be selected from 10bp; 20bp; 30bp; 40bp; 50bp; 60bp; 70bp; 80bp; 90bp; or 100bp.

10

In a further preferred embodiment of the invention said RNAi molecule comprises at least one of the nucleic acid sequences, or part thereof, presented in Figure 5.

15

In yet a further preferred embodiment of the invention said RNAi molecules comprise modified ribonucleotide bases.

It will be apparent to one skilled in the art that the inclusion of modified bases, as well as the naturally occurring bases cytosine, uracil, adenosine and guanosine, may confer advantageous properties on RNAi molecules containing said modified bases. For example, modified bases may increase the stability of the RNAi molecule thereby reducing the amount required to produce a desired effect.

20

25

According to a further aspect of the invention there is provided an RNAi molecule characterised in that it comprises the coding sequence of at least one gene which mediates at least one immunogenic response in at least one immune responsive cell.

In a preferred embodiment said coding sequence is an exon.

30

Alternatively said RNAi molecule is derived from intronic sequences or the 5' and/or 3' non-coding sequences which flank coding/exon sequences of genes which mediate said essential process.

- 5 In a further preferred embodiment of the invention the length of the RNAi molecule is between 10bp-1000bp. More preferably still when the length of RNAi is in the range of 100-1000bp the length is selected from 100bp; 200bp; 300bp; 400bp; 500bp; 600bp; 700bp; 800bp; 900bp; or 1000bp. More preferably still said RNAi is at least 1000bp. More preferably still when the length of RNAi is in the range of 10-1000bp the length is preferably from 10-100bp and may be selected from 10bp; 20bp; 30bp; 40bp; 50bp; 60bp; 70bp; 80bp; 90bp; or 100bp.

In a further preferred embodiment of the invention said RNAi molecule comprises at least one of the nucleic acid sequences, or part thereof, presented in Figure 1.

- 15 In yet a further preferred embodiment of the invention said RNAi molecules of Figure 1 comprise modified ribonucleotide bases.

- 20 It will be apparent to one skilled in the art that the inclusion of modified bases, as well as the naturally occurring bases cytosine, uracil, adenosine and guanosine, may confer advantageous properties on RNAi molecules containing said modified bases. For example, modified bases may increase the stability of the RNAi molecule thereby reducing the amount required to produce a desired effect.

- 25 A preferred method of the invention is to introduce to the cell a vector containing DNA which can encode a hairpin RNA. This is advantageous because, *inter alia*, when transcribed it allows the derivation of a cell stably expressing dsRNA or carrying an inducible expression cassette. Analogous methods are disclosed in our co-pending International Patent application WO 03/012082, which is incorporated
30 herein by reference.

It is known that short hairpin type of dsRNAs that are controlled by tRNA (Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Thus, the loop part of hairpin dsRNA, depending on its sequence, can target the dsRNA to different parts of the cells. One example of such targeting is the targeting of the dsRNA to the cytosol. More specifically, the sequence 5'-CUUCCUGUCA-3' appears to target the hairpin RNA correctly to the cytosol and appears to increase the efficiency of the RNAi effect.

10 According to a further aspect of the invention there is provided a nucleic acid sequence comprising at least part of a gene which mediates at least one essential process in a hyperproliferative cell wherein said nucleic acid is operably linked to at least one further nucleic acid sequence capable of promoting transcription of said nucleic acid linked thereto.

1.5 According to a further aspect of the invention there is provided a nucleic acid sequence comprising at least part of a gene which mediates at least one immunogenic response in a immune responsive cell wherein said nucleic acid is operably linked to at least one further nucleic acid sequence capable of promoting transcription of said nucleic acid linked thereto.

20 It will be apparent to one skilled in the art that the synthesis of RNA molecules which form RNAi can be achieved by providing vectors which include target genes, or fragments of target genes, operably linked to promoter sequences. Typically, promoter sequences are phage RNA polymerase promoters (e.g. T7, T3, SP6) if
25 RNAi molecules are produced *in vitro*. Advantageously vectors are provided with multiple cloning sites into which genes or gene fragments can be subcloned. Typically, vectors are engineered so that phage promoters flank multiple cloning sites containing the gene of interest. Phage promoters are oriented such that one promoter synthesises sense RNA and another promoter, antisense RNA. Thus, the synthesis of
30 RNAi is facilitated.

Target genes, or fragments of target genes, can be fused directly to promoters to create chimeric promoter/nucleic acid fusions via oligosynthesising technology. Constructs thus created can be easily amplified by polymerase chain reaction to provide templates for the manufacture of RNA molecules.

5

According to a further aspect of the invention there is provided a vector including a nucleic acid sequence comprising at least part of a gene which mediates at least one essential process in a hyperproliferative cell wherein said nucleic acid is operably linked to at least one further nucleic acid sequence capable of promoting transcription of said nucleic acid linked thereto.

10

According to a further aspect of the invention there is provided a vector including a nucleic acid sequence comprising at least part of a gene which mediates at least one immunogenic response in an immune responsive cell wherein said nucleic acid is operably linked to at least one further nucleic acid sequence capable of promoting transcription of said nucleic acid linked thereto.

15

If it is desired to form RNAi molecules *in situ*, expression vectors can be adapted to provide expression cassettes comprising nucleic acid molecules under the control of promoter sequences which result in the formation of sense and antisense RNA molecules. This may be facilitated by positioning promoter sequences upstream and downstream of said nucleic acid molecule such that both sense and antisense molecules are transcribed from the vector. Alternatively, separate expression cassettes can be adapted to produce sense and antisense RNAs.

20

25

Preferably said further nucleic acid sequence comprises a promoter sequence functional in a specific cell-type. Preferably the promoter sequence is functional in a keratinocyte, ideally a psoriatic cell or psoriatic keratinocyte.

Preferably said further nucleic acid sequence comprises a promoter sequence functional in a specific cell-type. Preferably the promoter sequence is functional in an immune responsive cell, ideally a stem, more ideally an embryonic stem cell.

- 5 In a preferred method of the invention said promoter sequences are selected from the following: keratin promoters K1; K5; K6; K10; K14; filaggrin; loricrin; involucrin.

Ideally said promoter sequence is keratin K6. It is known that the K6 promoter shows a high level of expression in epidermal cells undergoing hyperproliferation, see US
10 5,958,764.

It will be apparent to one skilled in the art that genes which show the requisite expression pattern are readily available. For example, and not by way of limitation, keratin K6, K5 and K14 are described in Woodcock and Mitchell, J. Cell Biol. 95,
15 p580-88 (1982); K1 and K10 are described in Roop et al Proc Natl. Acad.Sci USA, 80, p716-720, (1983) and Schweizer et al, Cell 37, p159-170, (1984). Many of these genes have been cloned and their sequences published, for example, K5, Lersch et al Mol Cell Biol. 8, p486-493, (1988); K14, Marchuk et al Proc.Natl.Acad.Sci.USA, 82, P1609-1613, (1985) and Knapp et al J.Biol Chem. 262, 938-945, (1987); K1,
20 Steinert et al., J.Biol.Chem. 260, p7142-7149, (1985); K10, Kreig et al J.Biol.Chem. 260, p5867-5870, (1985); K6, Tyner et al Proc.Natl Acad.Sci USA, 82, 4683-4687, (1985); loricrin, Yoneda et al. J.Biol.Chem. 267(25), 18060-18066.

Moreover, promoter sequences defining the 5' regions responsible for transcription
25 activation are known, for example see, Tomic et al Cell Reg. 1, p965-973 (K5, K6, K10, K14); Greenhalgh et al, Mol. Carcinogenesis, 7, p99-110, (1993). Methods for transfecting epidermal cells with vectors including epidermal specific promoters are also known as are methods relating to the heterologous expression of polypeptides in epidermal cells, see Morgan et al, Science, 237, p1476-1479, (1987); Teumer et al
30 FASEB, 4, p3245-3250, (1990); Sellheyer et al Proc.Natl.Acad.Sci USA, 90, p5237-

5241, (1993). Cell culture methods for keratinocytes are also known, see US5,968,546.

5 According to a further aspect of the invention there is provided a method to manufacture RNAi molecules comprising:

- (i) providing a vector or chimeric promoter/gene fusion;
- (ii) providing reagents and conditions which allow the synthesis of each RNA strand comprising said RNAi molecule; and
- (iii) providing conditions which allow each RNA strand to associate over at least part of their length, or at least that part corresponding to the nucleic acid sequence encoding a gene which mediates at least one essential process in a hyperproliferative cell.

15 According to a further aspect of the invention there is provided a method to manufacture RNAi molecules comprising:

- 20 (i) providing a vector or chimeric promoter/gene fusion;
- (ii) providing reagents and conditions which allow the synthesis of each RNA strand comprising said RNAi molecule; and
- (iv) providing conditions which allow each RNA strand to associate over at least part of their length, or at least that part corresponding to the nucleic acid sequence encoding a gene which mediates at least one immunogenic response in an immune responsive cell.

30 *In vitro* transcription of RNA is an established methodology. Kits are commercially available which provide vectors, ribonucleoside triphosphates, buffers, RNase

inhibitors, RNA polymerases (e.g. phage T7, T3, SP6) which facilitates the production of RNA.

5 According to a further aspect of the invention there is provided an *in vivo* method to inhibit an essential process in a hyperproliferative cell comprising administering to an animal an effective amount of RNAi according to the invention sufficient to inhibit cell proliferation.

10 It will be apparent to one skilled in the art that RNAi relies on homology between the target gene RNA and the RNAi molecule. This confers a significant degree of specificity to the RNAi molecule in targeting hyperproliferative cells.

15 According to a further aspect of the invention there is provided an *in vivo* method to inhibit an immunogenic response in an immune responsive cell comprising administering to an animal an effective amount of RNAi according to the invention sufficient to inhibit an immune response.

20 According to a further aspect of the invention there is provided an *in vitro* method to inhibit an immunogenic response in an immune responsive cell comprising treating said immune responsive cell with an effective amount of RNAi according to the invention sufficient to inhibit an immune response.

25 RNAi molecules may be encapsulated in liposomes to provide protection from an animals immune system and/or nucleases present in an animals serum.

30 Liposomes are lipid based vesicles which encapsulate a selected therapeutic agent which is then introduced into a patient. Typically, the liposome is manufactured either from pure phospholipid or a mixture of phospholipid and phosphoglyceride. Typically liposomes can be manufactured with diameters of less than 200nm, this enables them to be intravenously injected and able to pass through the pulmonary capillary bed. Furthermore the biochemical nature of liposomes confers permeability

across blood vessel membranes to gain access to selected tissues. Liposomes do have a relatively short half-life. So called STRALTH² liposomes have been developed which comprise liposomes coated in polyethylene glycol (PEG). The PEG treated liposomes have a significantly increased half-life when administered intravenously to a patient. In addition STRALTH² liposomes show reduced uptake in the reticuloendothelial system and enhanced accumulation selected tissues. In addition, so called immuno-liposomes have been develop which combine lipid based vesicles with an antibody or antibodies, to increase the specificity of the delivery of the RNAi molecule to a selected cell/tissue.

10

The use of liposomes as delivery means is described in US 5580575 and US 5542935 which are incorporated by reference.

It will be apparent to one skilled in the art that the RNAi molecules can be provided in the form of an oral or nasal spray, an aerosol, suspension, emulsion, and/or eye drop fluid. Alternatively the RNAi molecules may be provided in tablet form. Alternative delivery means include inhalers or nebulisers.

15

According to a yet further aspect of the invention there is provided a therapeutic composition comprising at least one RNAi molecule according to the invention. Preferably said RNAi molecule is for use in the treatment of hyperproliferative diseases. More preferably still the hyperproliferative disease is psoriasis

20

According to a yet further aspect of the invention there is provided a therapeutic composition comprising at least one RNAi molecule according to the invention. Preferably said RNAi molecule is for use in the treatment of conditions which would benefit from immunosuppression. More preferably still the condition is transplant rejection.

25

In a further preferred embodiment of the invention said therapeutic composition further comprises a diluent, carrier or excipient.

30

Embodiments of the invention will now be described by example only and with reference to the following materials and methods.

5 MATERIALS AND METHODS

Cell Culture Conditions

10 Normal human keratinocytes (NHK) were isolated from skin after a punch biopsy and were then maintained in Keratinocyte-SFM.

NTERA2 cLD1 and 2102Ep cl.2A6 human EC cells were cultured in DMEM containing 10% heat-inactivated FCS, as previously described. The human ES cell line, H7 (ref 1) was cultured in 'Knock-Out'-DMEM (Invitrogen) supplemented with
15 20% Serum Replacement (Invitrogen) and 4 ng/ml bFGF (Invitrogen), on feeder layers of mouse embryonic fibroblasts mitotically inactivated with Mitomycin C^{1,16}. For stable transfection, the cells were seeded at 3×10^4 per cm² and transfected one day later with pCAG-GFP using Exgen 500 (Fermentas) following the manufacturer's protocol. Puromycin (1 µg/ml) was added to the cultures, 24 hours
20 after transfection, and puromycin-resistant green-fluorescent colonies were selected and expanded for further experiments.

RNAi Production and Treatment of Cells *in vitro*

25 DNA fragments corresponding to specific gene sequences were amplified by PCR (Mullis and Faloona, 1987) to generate specific templates for RNAi production. Both primers used in a particular reaction contained a 5' T7 RNA polymerase binding site (TAATACGACTCACTATAGGGAGA) appended to gene specific sequences. PCR reactions were electrophoresed in 1% agarose and the single band at
30 the correct size for the amplification was excised and purified. 5µl of a 100µl PCR

reaction was used as a template for a MEGASCRIP T7 transcription reaction (Ambion, USA). The products from the MEGASCRIP reaction were precipitated and re-suspended in water. The RNAi was created by heating the MEGASCRIP reactions to 65°C for 30 minutes and cooling to room temperature to allow annealing.

- 5 To test for production of full length dsRNA 6µg was electrophoresed on a 1% denaturing agarose gel and stained with ethidium bromide. The RNA was observed by transillumination with 302nm UV light.

- 10 The following method describes RNAi of cells cultured in 6 well plates. Volumes and cell numbers should be scaled appropriately for larger or smaller culture vessels.

- Cells were seeded at 500,000 per well on the day prior to treatment and grown in their normal medium. For each well to be treated, 9.5µg of the double stranded RNA of interest was diluted in 300µl of 150mM NaCl. 21µl of ExGen 500 (MBI
15 Fermentas) was added to the diluted RNA solution and mixed by vortexing. The dsRNA/ExGen 500 mixture was incubated at room temperature for 10 minutes. 3ml of fresh cell growth medium was then added, producing the RNAi treatment medium. Growth medium was aspirated from the culture vessel and replaced with 3ml of RNAi treatment medium per well. Culture vessels were then centrifuged at 280g for
20 5 minutes and returned to the incubator. After 12-18hrs, RNAi treatment medium was replaced with normal growth medium and the cells maintained as required.

siRNA Production and Treatment of EC and ES cells

- 25 siRNAs corresponding to *eGFP*, *β2M* and *Oct4* were designed with the following sense and antisense sequences and were synthesized by Xeragon Inc. (Huntsville, USA).

eGFP: 5' - CGUAAACGGCCACAAGUUCdTdT - 3' (sense) and,

5' - GAACUUGUGCCCGUUUACGdTdT - 3' (antisense);

β2M: 5' -GAUUCAGGUUUACUCACGUdTdT-3' (sense) and,

5' -ACGUGAGUAAACCUGAAUCdTdT-3' (antisense);

Oct4: 5' -AGCAGCTUGGGCUCGAGAAdTdT-3' (sense) and,

5' -UUCUCGAGCCCAAGCUGCUdTdT-3' (antisense).

Cells were trypsinised and plated in 6 well plates at 2×10^4 per cm^2 . The next day, siRNA was introduced into the cells using the Oligofectamine transfection reagent (Invitrogen) following the manufacturer's protocol. In brief, 10 μl siRNA (20 μM solution) was incubated with 4 μl Oligofectamine in 190 μl Optimem (Invitrogen) for 20 min; the mixture was then added to the cells in a final volume of 1.2 ml. The transfected cells were cultured and were fed daily with fresh medium until assayed.

15

RNA Extraction

Growing cultures of cells were aspirated to remove the DME and foetal calf serum. Trace amounts of foetal calf serum was removed by washing in Phosphate-buffered saline. Fresh PBS was added to the cells and the cells were dislodged from the culture vessel using acid washed glass beads. The resulting cell suspension was centrifuged at 300xg. The pellets had the PBS aspirated from them. Tri reagent (Sigma, USA) was added at 1ml per 10^7 cells and allowed to stand for 10 mins at room temperature. The lysate from this reaction was centrifuged at 12000 x g for 15 minutes at 4°C. The resulting aqueous phase was transferred to a fresh vessel and 0.5 ml of isopropanol / ml of trizol was added to precipitate the RNA. The RNA was pelleted by centrifugation at 12000 x g for 10 mins at 4°C. The supernatant was removed and the pellet washed in 70% ethanol. The washed RNA was dissolved in DEPC treated double-distilled water.

25

Analysis of Transfectants by Antibody Staining and FACS

Cells were treated with trypsin (0.25% v/v) for 5 mins to disaggregate the cells; they
5 were washed and re-suspended to 2×10^6 cells/ml. This cell suspension was incubated
with 50 μ l of primary antibody in a 96 well plate on a rotary shaker for 1 hour at 4°C.
Supernatant from a myeloma cell line P3X63Ag8, was used as a negative control.
The 96 well plate was centrifuged at 100rpm for 3 minutes. The plate was washed 3
10 times with PBS containing 5% foetal calf serum to remove unbound antibody. Cell
were then incubated with 50 μ l of an appropriate FITC-conjugated secondary
antibody at 4°C for 1 hour. Cells were washed 3 times in PBS + 5% foetal calf
serum and analysed using an EPICS elite ESP flow cytometer (Coulter electronics,
U.K.) (Andrews et. al., 1982).

15 Analysis of Antigen Expression on EC and ES cells by Immunofluorescence and FACS

Antigen expression was assayed by immunofluorescence and flow cytometry as
previously described (Draper et. al., 2002) using the following monoclonal antibodies:
20 MC631, anti-stage specific embryonic antigen-3 (SSEA3); MC813-70, anti-stage
specific embryonic antigen-4 (SSEA4); MC480, anti-stage specific embryonic
antigen-1 (SSEA1); TRA-1-60; TRA-2-54, anti-liver/kidney/bone alkaline
phosphatase; BBM1; W6/32.

25

Northern Blot Analysis of RNA

RNA separation relies on the generally the same principles as standard DNA but with
some concessions to the tendency of RNA to hybridise with itself or other RNA
molecules. Formaldehyde is used in the gel matrix to react with the amine groups of
30 the RNA and form Schiff bases. Purified RNA is run out using standard agarose gel
electrophoresis. For most RNA a 1% agarose gel is sufficient. The agarose is made
in 1X MOPS buffer and supplemented with 0.66M formaldehyde. Dried down RNA

samples are reconstituted and denatured in RNA loading buffer and loaded into the gel. Gels are run out for approx. 3 hrs (until the dye front is 3/4 of the way down the gel).

- 5 The major problem with obtaining clean blotting using RNA is the presence of formaldehyde. The run out gel was soaked in distilled water for 20 mins with 4 changes, to remove the formaldehyde from the matrix. The transfer assembly was assembled in exactly the same fashion as for DNA (Southern) blotting. The transfer buffer used however was 10X SSPE. Gels were transferred overnight. The membrane
- 10 was soaked in 2X SSPE to remove any agarose from the transfer assembly and the RNA was fixed to the membrane. Fixation was achieved using short-wave (254 nm) UV light. The fixed membrane was baked for 1-2 hrs to drive off any residual formaldehyde.
- 15 Hybridisation was achieved in aqueous phase with formamide to lower the hybridisation temperatures for a given probe. RNA blots were prehybridised for 2-4 hrs in northern prehybridisation solution. Labelled DNA probes were denatured at 95°C for 5 mins and added to the blots. All hybridisation steps were carried out in rolling bottles in incubation ovens. Probes were hybridised overnight for at least 16
- 20 hrs in the prehybridisation solution. A standard set of wash solutions were used. Stringency of washing was achieved by the use of lower salt containing wash buffers. The following wash procedure is outlined as follows

	2X SSPE	15 mins	room temp
	2X SSPE	15 mins	room temp
25	2X SSPE/ 0.1% SDS	45 mins	65°C
	2X SSPE/ 0.1% SDS	45 mins	65°C
	0.1X SSPE	15 mins	room temp

Preparation of radiolabelled DNA probes

- 30 The method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983) was used to radioactively label DNA. Briefly, the protocol uses random sequence hexanucleotides

- to prime DNA synthesis at numerous sites on a denatured DNA template using the Klenow DNA polymerase I fragment. Pre-formed kits were used to aid consistency. 5-100ng DNA fragment (obtained from gel purification of PCR or restriction digests) was made up in water, denatured for 5 mins at 95°C with the random hexamers. The mixture was quench cooled on ice and the following were added,

5 µl [α -32P] dATP 3000 Ci/mmol
1 µl of Klenow DNA polymerase (4U)

- 10 The reaction was then incubated at 37°C for 1 hr. Unincorporated nucleotide were removed with spin columns (Nucleon Biosciences).

Production of cDNA

- 15 The enzymatic conversion of RNA into single stranded cDNA was achieved using the 3' to 5' polymerase activity of recombinant Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase primed with oligo (dT) and (dN) primers. For Reverse Transcription-Polymerase Chain Reaction, single stranded cDNA was used. cDNA was synthesised from 1µg poly (A)+ RNA or total RNA was incubated with the following

- 20 1.0µM oligo(dT) primer for total RNA or random hexamers for mRNA
0.5mM 10mM dNTP mix
1U/µl RNase inhibitor (Promega)
1.0U/µl M-MLV reverse transcriptase in manufacturers supplied buffer (Promega)

- 25 The reaction was incubated for 2-3 hours at 42°C

Fluorescent Automated Sequencing

- 30 To check the specificity of the PCR primers used to generate the template used in RNAi production automatic sequencing was carried out using the prism fluorescently

labelled chain terminator sequencing kit (Perkin-Elmer) (Prober et al 1987). A suitable amount of template (200ng plasmid, 100ng PCR product), 10 μ M sequencing primer (typically a 20mer with 50% G-C content) were added to 8 μ l of prism pre-mix and the total reaction volume made up to 20 μ l. 24 cycles of PCR (94°C for 10 seconds, 50°C for 10 seconds, 60°C for 4 minutes). Following thermal cycling, products were precipitated by the addition of 2 μ l of 3M sodium acetate and 50 μ l of 100 % ethanol. DNA was pelleted in an Eppendorf microcentrifuge at 13000 rpm, washed once in 70% ethanol and vacuum dried. Samples were analysed by the in-house sequencing Service (Krebs Institute). Dried down samples were resuspended in 4 μ l of formamide loading buffer, denatured and loaded onto a ABI 373 automatic sequencer. Raw sequence was collected and analysed using the ABI prism software and the results were supplied in the form of analysed histogram traces.

Reverse transcription and polymerase chain reaction for determining the expression of genes in EC and ES cells.

Total RNA (2 μ g) was reverse transcribed using 1 μ g oligo-dT primer with MMLV Reverse-Transcriptase (Promega) in a 40 μ l reaction volume containing 1.25 mM dNTPs at 37°C. Oligonucleotide primers for use in PCR were designed using the PrimerSelect program from the DNASTAR software package (DNASTAR Inc., Madison, WI). PCR was performed using 1 μ l of cDNA in 25 μ l PCR containing 15 pmol of each primer, 0.1 mM dNTPs and 0.3 units *Taq* polymerase (Promega). Primer sequences used and conditions of these reactions were as follows:

hCG-f: 5' -ATGGGCGGGACATGGGCATCCA-3', (70°C annealing, x35 cycles);

hCG-r: 5' -GGCCCCGGGAGTCGGGATGG-3', (70°C annealing, x35 cycles);

Cdx2-f: 5' -CCTCCGCTGGGCTTCATTCC-3', (60°C annealing, x30 cycles);

Cdx2-r: 5' -TGGGGGTTCTGCAGTCTTTGGTC-3' (60°C annealing, x30 cycles);

β-actin-f: 5' -ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' (70°C annealing, x25 cycles);

5 *β-actin-r*: 5' -CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' (70°C annealing, x25 cycles).

Detection of specific protein targets by SDS-PAGE and Western Blotting

10 To obtain cell lysates monolayers of cells were rinsed 3 times with ice-cold PBS supplemented with 2 mM CaCl₂. Cells were incubated with 1 ml/75 cm² flask lysis buffer (1% v/v NP40, 1% v/v DOC, 0.1 mM PMSF in PBS) for 15 min at 4°C. Cell lysates were transferred to eppendorf tubes and passed through a 21 gauge needle to shear the DNA. This was followed by freeze thawing and subsequent centrifugation (30 min, 4°C, 15000g) to remove insoluble material. Protein concentrations of the
15 supernatants were determined using a commercial protein assay (Biorad) and were adjusted to 1.3 mg/ml. Samples were prepared for SDS-PAGE by adding 4 times Laemmli electrophoresis sample buffer and boiling for 5 min. After electrophoresis with 16 µg of protein on a 10% polyacrylamide gel (Laemmli, 1970) the proteins were transferred to nitro-cellulose membrane with a pore size of 0.45µm. The blots
20 were washed with PBS and 0.05% Tween (PBS-T). Blocking of the blots occurred in 5% milk powder in PBS-T (60 min, at RT). Blots were incubated with the appropriate primary antibody. Horseradish peroxidase labelled secondary antibody was used to visualise antibody binding by ECL (Amersham, Bucks., UK). Materials used for SDS-PAGE and western blotting were obtained from Biorad (California,
25 USA) unless stated otherwise.

Detection of specific protein targets on EC and ES by SDS-PAGE and Western Blotting	
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5

Cells were harvested using trypsin and lysed in RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) at 10^7 cells/ml. These lysates were electrophoresed using SDS-PAGE on a 10% polyacrylamide gel and blotted overnight onto PVDF membrane. Membranes were stained with Ponceau-S to check loading, and blocked for 1 hr with 5% fat-free milk solution. Samples were probed with a goat polyclonal antibody against Oct4 (Santa-Cruz Biotechnology) at a concentration of 0.25 μ g/ml and a 1:4000 Dilution of anti-goat IgG-peroxidase conjugate (Sigma-Aldrich). Staining was visualized using ECL kit (Amersham Biosciences).

15

RESULTS

The effect of RNAi on *Ocd4* protein expression and on levels of cell proliferation and differentiation.

20

The nullipotent EC line, 2102Ep, was treated with siRNA targeting *Oct4*. The levels of *Oct4* protein fell markedly as detected by Western blotting (Figure 1a). The maximum effect on *Oct4* protein level was seen between 3 and 5 days after treatment with siRNA, and the level began to rise again by 8 days. β 2-microglobulin siRNA, used as a control, had no effect on *Oct4* mRNA and protein level.

Cells treated with *Ocr4* siRNA grew considerably more slowly (Figure 3) and acquired a large flat morphology indicative of differentiation (Figure 1b).

To test the effect of RNAi-mediated knock down of Oct4 expression, 2102Ep and NTERA2 human EC, and H7 human ES cells were treated with siRNA targeting *Oct4* and $\beta 2M$ for 3 to 5 days. A marked, specific reduction in Oct4 protein level was observed in each cell line (Figure 2a) and significant populations of SSEA3-negative and TRA-1-60 negative cells appeared (Figure 2b). Loss of these characteristic markers of human EC and ES cells is an early indication of differentiation. The appearance of these antigen-negative cells was most obvious in the *Oct4* siRNA-treated EC lines, because their cultures showed less tendency to differentiate spontaneously in the controls compared to the ES cells, cultures of which typically contained significant numbers of spontaneously differentiated cells even under optimal conditions. Nevertheless, it was clear that *Oct4* RNAi induced the same changes in the ES cells as in the EC cells. At the same time, the number of SSEA1-positive cells, also indicative of differentiation, increased in the 2102Ep EC and H7 ES cultures treated with *Oct4* siRNA, although, only a limited appearance of SSEA1-positive cells was noted in the NTERA2 cultures. Again, as expected, $\beta 2M$ siRNA caused a marked down-regulation of $\beta 2M$ expression in 2102Ep and H7 cells compared with cells treated with *Oct4* siRNA. In this experiment, little $\beta 2$ -microglobulin expression was seen in the NTERA2 cultures in either case, but this accords with past experience that NTERA2 cells frequently express only very low, barely detectable levels of $\beta 2M$ and HLA, whereas expression of both is regularly observable in 2102Ep and H7 cells.

Exposure of malignant EC cells, 2102, 1777 and Tera-1 to Oct4 RNAi caused a marked reduction in cell growth (Figure 3).

Oct4 siRNA treatment, *hCG* mRNA was significantly up-regulated in 2102Ep and H7 cells (Figure 4) indicating that differentiation into trophectoderm is a consequence of lowered Oct4 levels in human EC and ES cells. Consistent with this,

the transcription factor *Cdx2*, associated with this differentiation lineage in the mouse (Rossant and Cross, 2001), was also up-regulated in 2102Ep cells (Figure 4).

However, *Cdx2* was already expressed at significant levels in the control H7 cells, and no further change was noted after siRNA treatment. This probably reflects the tendency of these pluripotent cells to differentiate spontaneously to some extent in the control cultures. By contrast, NTERA2 cells behaved differently. Indeed, a weak and barely detectable band corresponding to *hCG* did appear in the cells treated with *Oct4* siRNA but, although *Cdx2* was expressed in the control cells, it was down-regulated following treatment with *Oct4* siRNA.

Treatment of the cells with siRNA to $\beta 2M$

Treatment of the cells with siRNA to $\beta 2M$ resulted in a substantial reduction in the binding of antibody BBM1 to $\beta 2$ -microglobulin and the HLA-A,B,C heavy chain which requires $\beta 2$ -microglobulin for cell surface expression (Arce-Gomez et. al., 1978) as analysed by flow cytometry.

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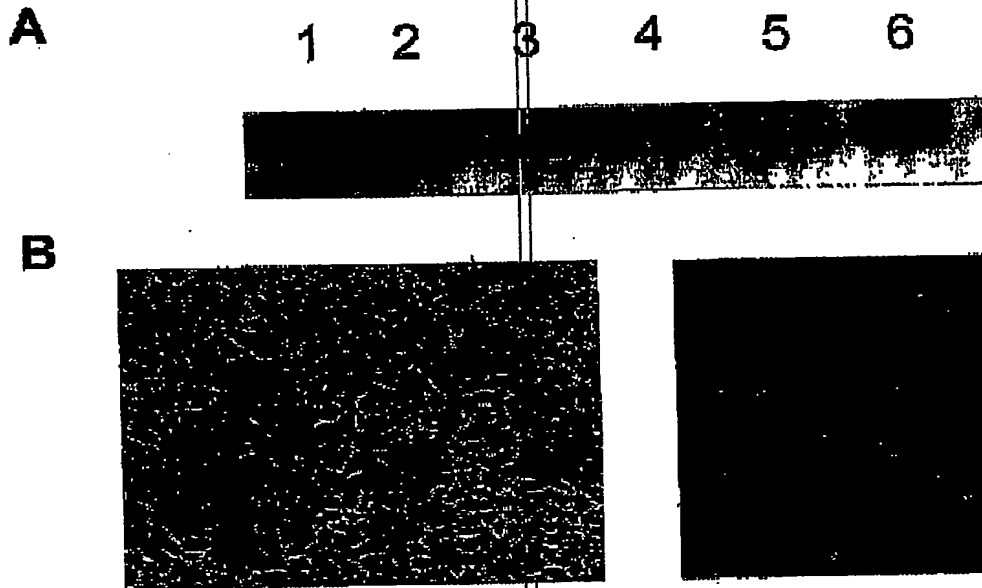
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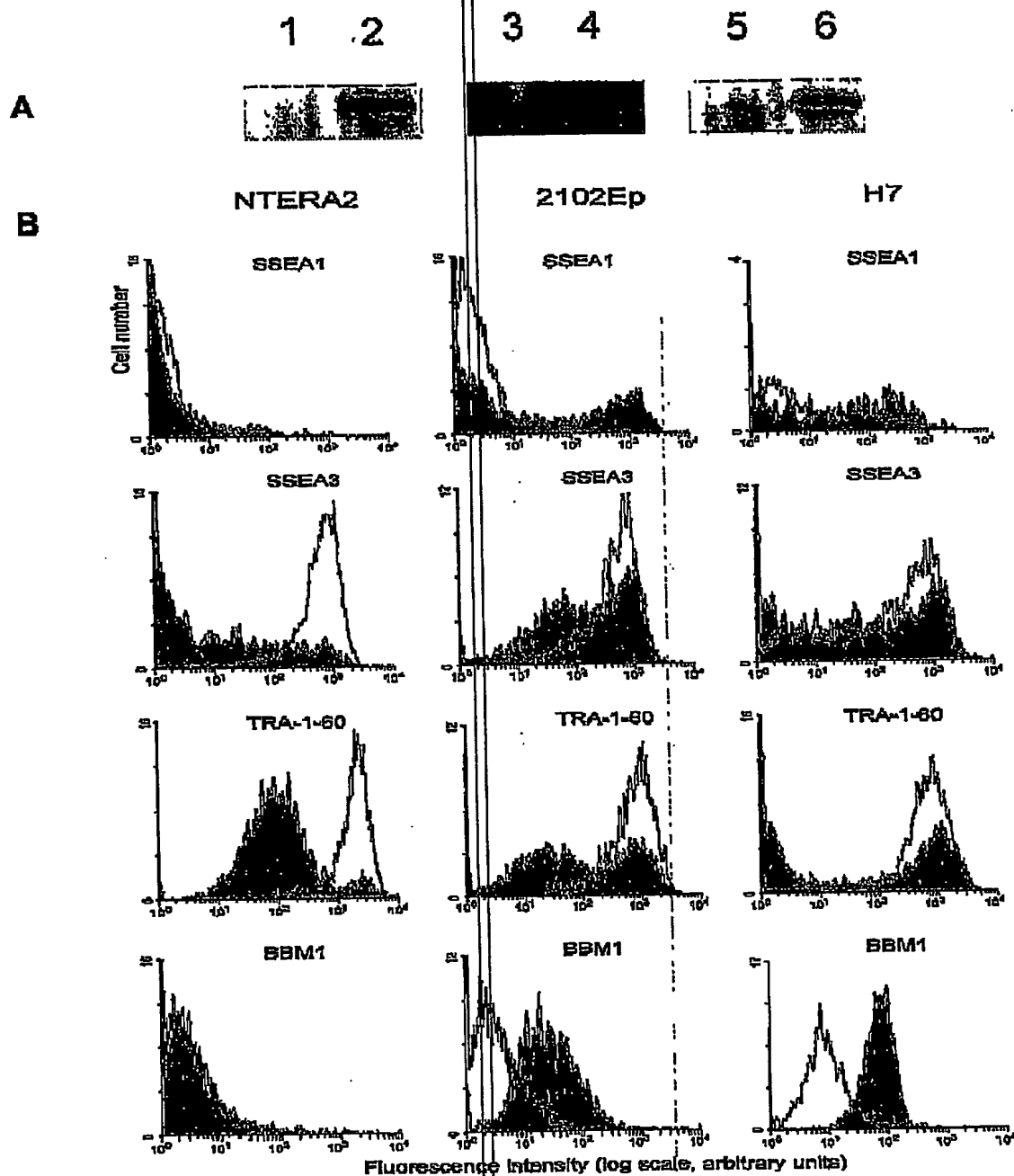
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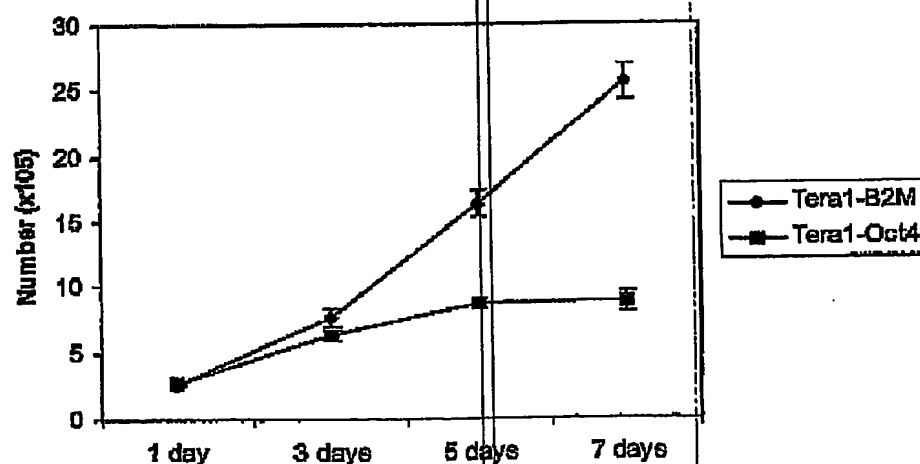
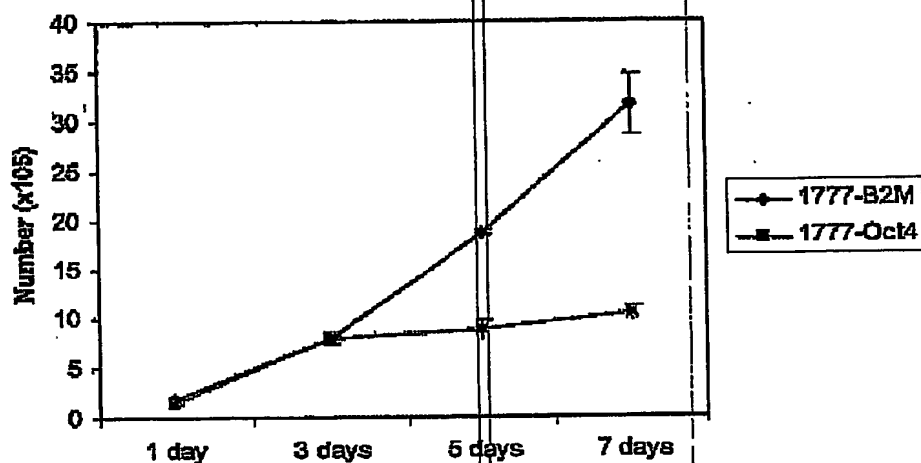
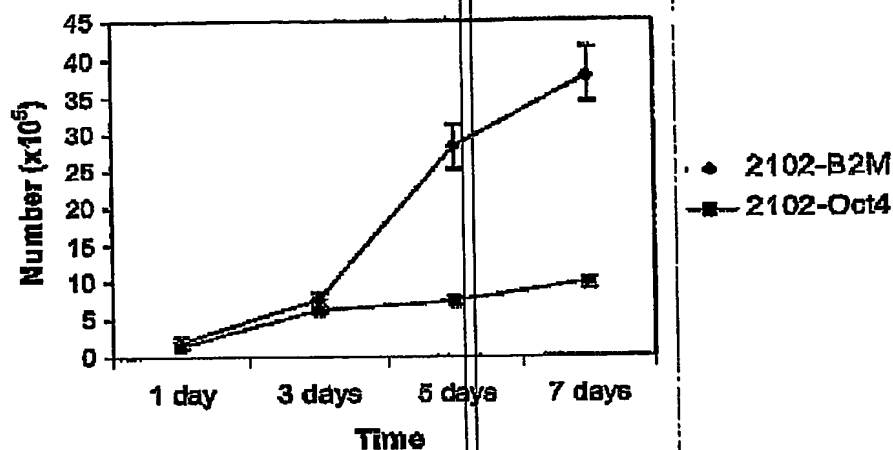
Figure 1:A: Western blot for Oct-4 expression in cells treated with dsRNA targeting either 2M (Lanes 1, 3, 5) or Oct4 (Lanes 2, 4, 6) after 3 days (Lanes 1, 2), 5 days (Lanes 3, 4) or 7 days (Lanes 5, 6). B: Morphology of 2102Ep cells, 7 days after treating with siRNA targeting b2M (panel A) and Oct-4 (panel B).

2/8



5 **Figure 2:** NTERA2 and 2102Ep EC cells and H7 ES cells were treated with dsRNAs targeting Oct-4, and β2M and analysed 5 days later. a, Western blot for Oct-4 protein in NTERA2 (lanes 1, 2), 2102Ep (lanes 3, 4) and H7 (lanes 5, 6); siRNA to Oct-4 (lanes 1, 3 and 5; siRNA to 2M (lanes 2, 4 and 6). B: Surface antigen expression after treatment with siRNA targeting Oct-4 (shaded histograms) and β2M (non-shaded histograms).

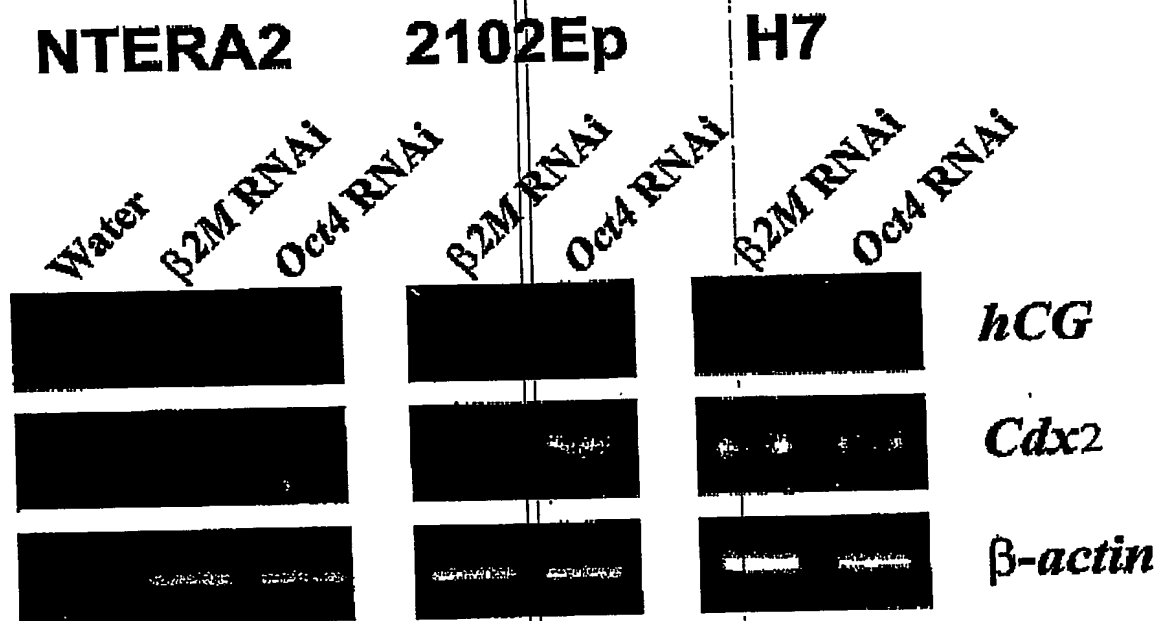
3/8



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Figure 3: Cells were harvested and counted from triplicate wells that had originally been seeded with 2×10^5 cells one day before treatment with dsRNA to -b2M and Oct-4 on day 0.

5



- 10 **Figure 4:** After treatment with dsRNAs targeting Oct4 and 2M, the expression of hCG and Cdx2 were examined by RT-PCR. β -actin PCR was used as a template loading control. In response to Oct-4 RNAi, the trophoblast-specific mRNA, hCG, was induced in 2102Ep and H7 cells, and Cdx2, which encodes a transcription factor associated with trophectoderm differentiation, was up-regulated in 2102Ep but down-regulated in NTERA2 cells. Cdx2 was expressed appreciably in control H7 cells and no significant change was seen after treatment with siRNA to Oct-4.
- 15

20

F038154.4

31

Fig. 5

Oct4

>gi|4505966|ref|NM_002701.1| Homo sapiens POU domain, class 5, transcription factor 1 (POU5F1), mRNA
 GTAGTCCTTTGTTACATGCATGAGTCAGTGAACAGGGAATGGGTGAATGACATTTGTGGGTAGGTTATTT
 CTAGAAGTTAGGTGGGCAGCTCGGAAGGCAGATGCACCTTCTACAGACTATTCTTGGGGCCACACGTTAGG
 TTCTTGAATCCCGAATGGAAAGGGGAGATTGATAACTGGTGTGTTTATGTTCTTACAAAGTCTTCTGCCTT
 TTAATAATCCAGTCCAGGACATCAAAAGCTCTGCAGAAAGAACTCGAGCAATTTGCCAAGCTCTGAAAGCA
 GAAGAGGATCACCTGGGATATACACAGGCCGATGTGGGGCTCACCTTGGGGGTTCTATTTGGGAAGGTA
 TTCAGCCAAACGACCATCTGCCGCTTTGAGGCTCTGCAGCTTAGCTTCAAGACATGTGTAGCTGCGGC
 CCTTGCTGCAGAAAGTGGGTGGAGGAAGCTGACACCAATGAAAATCTTCAGGAGATATGCAAGCAGAAAC
 CCTCGTGCAGGCCCGAAAGAGAAAGCCAAACCATATCGAGAACCAGTGAAGAGGCAACCTGGAGAATTG
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 AGAGGATTTTGAAGCTGCTGGGTCTCCTTTCTCAGGGGGACAGTGTCTTCTCTGCCCCAGGGCCC
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 GCCCTTCTAGGAATGGGGACAGGGGGAGGGGAGAGCTAGGGAAAGAAAACCTGGAGTTTGTGCCAGGG
 TTTTGGGATTAAGTCTTCACTTCACTAAGGAAGGAATTGGGAACACAAAGGGTGGGGGCAGGGGAGTTG
 GGCACACTGGTTGGAGGGAAGGTGAAGTTCAATGATGCTCTTGATTTTAATCCACATCATGTATCACTT
 TTTTCTTAAATAAAGAAGCTTGGGACACAGTAGATAGA

Beta-2-Microglobulin

>gi|4757825|ref|NM_004048.1| Homo sapiens beta-2-microglobulin (B2M), mRNA
 GGCACGAGCCGAGATGTCTCGCTCCGTGGGCTTAACTGTGCTCGGCTACTCTCTCTTTCTGGCCTGGAG
 GCTATCCAGCGTACTCCAAAGATTCAAGTTTACTTCACGTATCCAGCAGAGAATGGAAAGTCAATTTTCC
 TGAATTGCTATGTGTCTGGGTTTCATCCATCCGACATTTGAAGTTGACTTACTGAGAGAATGGAGAGAGAAT
 TGAATAAGTGGAGCATTCAGACTTGTCTTTCAAGCAAGGACTGGTCTTTCTATCTCTTGTACTACACTGAA
 TTCACCCCACTGAAAAAGATGAGTATGCCTGCCGTGTGAACCATGTGACTTTGTACAGCCCAAGATAG
 TTAAGTGGGATCGAGACATGTAAGCAGCATCATGGAGCTTTGAAGATGCCCATTTGCAATTGGATGAATT
 CCAATTTCTGCTTGCCTTCTTTTAAATATTGATATGCTTATACACTTACACTTTATGCACAAATGTAGG
 GTTATAATTAATGTTAAGATGGACATGATCTTCTTTATAATTTCTACTTTGAGTGTCTGTCTCCATGTTTGAT
 GTATCTGAGCAGGTTGCTCCACAGGTAGCTCTAGGAGGCTGGCAACTTAGAGGTGGGGAGCAGAGAATT
 CTCTTATCCAACATCAACATCTTGGTCAAGATTGAAGTCTTCAATCTCTTGCACCTCAAAGCTTGTAAAGA
 TAGTTAAGCGTGCATAAGTTAAGTTCAATTTACATACTCTGCTTGAATTTGGGGGAAAATTTAGAAAT
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 TTCTTATACATTTGATAAGTAAGGCATGCTTGTGGTTAATCTGCTTATTTTGTCTCCACAAGTTAAAT
 AATCATAAACTTG

HLA-A

>gi|24797066|ref|NM_002116.4| Homo sapiens major histocompatibility complex, class I, A (HLA-A), mRNA
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 CGGGCTCCCACTCCATGAGGTAATTTCTTACATCGGTGTCCCGCCCGGGCCGGGGAGCCCCGCTTCAT
 CGCCGTGGGCTACGTGGACGACACGCAGTTCTGTCGGTTCCACAGCGACGCCGAGCCAGAGGATGGAG
 CCGCGGGCCCGTGGATAGAGCAGGAGGGGCCGAGTATTGGGACCAAGGAGACACGGAAATGTGAGGCCCC
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ACACATATGACCCACCCACCCCATCTCTGACCATGAGGCCACCCCTGAGGTGCTGGGCGCTGGGCTTCTACC
CTGCGGAGATCACACTGACCTGGCAGCGGGATGCGGAGGACCAAGCCACGACACCGGAGCTCGTGGAGAC
CAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGCGGCTGTGGTGGTGGCTTCTGGAGAGGAGCAGAGA
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CCACCATCCCCATCGTGGGCATCATTTGCTGGCTGGTTCTCTCTTGGAGCTGTGATCACTGGAGCTGTGGT
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GACAGTGGCCAGGGCTCTGATGTGTCTCCCTCACAGCTTGTAAAGTGTGA

HLA-B

>gi|21327676|ref|NM_005514.4| Homo sapiens major histocompatibility
complex, class I, B (HLA-B), mRNA
AGACGCGGAGATGCTGGTCACTGGCGCCCCGAACCGTCTCTCTGCTGCTCTCGGCGGCGCTTGGCCCTGACC
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TGGAGGCGGAGTGGCTGGAGTGGCTCCCGCAGATACCTGGAGAACCGGAAGGACAAGCTGGAGCGCGCTGA
CCCCCAAGAGACACACGTTGACCCAGCAGCCCATCTCTGACCATGAGGCCACCTGAGGTGCTGGGCGCTG
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HLA-C

>gi|19557676|ref|NM_002117.3| Homo sapiens major histocompatibility
complex, class I, C (HLA-C), mRNA
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GGAGCCCCGCTTCATCGCAGTGGGCTACGTGGACGACACGCACTTCTGCGGTTCCAGACGACGCGCGG
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CCCACCCCGTGTCCACCGTGACCCCTGTCCCGACACTGACCTGTGTTCCTCCCGATCATCTTTCTGT
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CCCGAATTC

HLA-E

>gi|24797070|ref|NM_005516.3| Homo sapiens major histocompatibility
complex, class I, E (HLA-E), mRNA

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TCACACTGACCTGGCAGCAGGATGGGGAGGGCCATACCCAGGACACGGAGCTCGTGGAGACCAGGCTGTC
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GAGTGGAGCGACAGTCCCAGGGGTCTGAGTCTCACAGCTTGTA

HLA-F

>gi|9665231|ref|NM_018950.1| Homo sapiens major histocompatibility
complex, class I, F (HLA-F), mRNA

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TCACGCTGACCTGGCAGCGGGATGGGGAGGAACAGACCCAGGACACAGAGCTTGTGGAGACCAAGCCCTGC
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CATGTGCAGCAGAGGGGCTGCCCGAGCCCTCTATCTGAGATGGGAGCAGTCTCCCCAGCCCAACCATCC
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CAGGGCTCTGGGCTGTCTCTCACAGCTAATAAAGTGTGAGACAGCTTCTTGTGTGGGACTGAGAAGCAA
GATATCAATGTAGCAGAAATGCACTTGTGCGCTCAAGACATACATAAATTTAAAAATAAAGAAATAA

818

HLA-G

>gi|24797072|ref|NM_002127.3| Homo sapiens HLA-G histocompatibility antigen, class I, G (HLA-G), mRNA

CCCATTAGGTGACAGGTTTTTTAGAGAAAGCCAAATCACGTGCGCGCGGTCTCTGTTCTAAAGTCCTCGCTCA
CCCACCCCGGACTCATTCTCCCCAGACGCCAAGGATGSGTGTCTATGCGCCCCGAACCCCTCTTCCTGCTGC
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TTCGACAGCGACTCGGCGTGTCCGAGGATGGAGCCGCGGGCGCGCTGGGTGGAGCAGGAGGGGCGCGAGT
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CGGCTACTACAACAGAGCGAGGCCAGTTCTCACACCCCTCCAGTGGATGATTGGCTGCGACCTGGGGTCC
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